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High-Pressure Inactivation and Sublethal Injury of Pressure-Resistant *Escherichia coli* Mutants in Fruit Juices

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The potential of high-pressure-resistant mutants of *Escherichia coli* to survive high-pressure pasteurization in fruit juices and in low-pH buffers was investigated. Treatments with up to 500 MPa of pressure caused only a limited direct inactivation of the mutants but resulted in an accelerated low-pH inactivation during subsequent storage.

High hydrostatic pressure can be used to inactivate microorganisms and quality-deteriorating enzymes in foods (6, 7), and, at least in some foods, like fruit juices, this process allows a better retention of the original flavor and taste than does thermal treatment (9–12). Pressure-treated-fruit-based foods were first introduced on the Japanese market in 1990, and it is likely that they will also be among the first such products introduced in Europe and the United States. Besides good retention of flavor, an important reason for using this process is that the low pH of fruit products (pH 3 to 4) does not support growth of pathogenic bacteria which may eventually survive pressurization. We recently succeeded in the isolation of spontaneous extremely pressure-resistant mutants from a pressure-sensitive *Escherichia coli* strain (5). Based on studies in phosphate buffer, we anticipated that these mutants would be able to survive pasteurization of food products at very high pressures (800 MPa or more) and mild temperatures (10 to 40°C). Although it is not yet clear whether pressure resistance occurs naturally in strains of *E. coli* or other bacteria, the possibility that pressure-resistant strains will build up in the selective environment of a high-pressure (HP) food processing plant cannot be excluded.

Although they cannot grow at low pH, some strains of *Enterobacteriaceae*, like certain strains of *E. coli* and *Shigella* and *Salmonella* species, can survive for several days or even weeks in acidic foods (3, 8, 13). This was particularly well documented for *E. coli* O157:H7 after this organism was implicated in a number of recent infectious outbreaks caused by consumption of unpasteurized apple juice and cider (1, 2, 4, 14). Clearly, the efficient inactivation of *E. coli* will be a primary and nonnegotiable requirement for HP processes for the production of high-quality and safe fruit juices. Therefore, the aim of the present work was to study the HP inactivation of pressure-resistant *E. coli* mutants in three different fruit juices and in low-pH buffers.

MG1655, LMM1010, LMM1020, and LMM1030 are the parental *E. coli* strain and three different pressure-resistant mutants thereof, respectively (5). Cultures in Luria-Bertani broth grown to stationary phase at 37°C for 21 h with shaking were harvested by centrifugation (3,000 × *g*), and the cells were resuspended in juice or in 50 mM HEPES buffer. Pressurizations were done for 15 min at 20°C on small samples (0.5 to 1 ml) sealed in sterile polyethylene bags. The temperature increase due to adiabatic compression never exceeded 10°C. When survival had to be monitored during storage after HP treatment, the number of replicate bags prepared and simultaneously treated was sufficient to allow destructive sampling. All data shown in the figures and table below are representative results from three independent experiments. Survivors were enumerated by performing plate counts on tryptic soy agar (Biokar Diagnostics, Beauvais, France).

As a preliminary experiment, we studied the long-term survival of the strains in the three types of juice at two different storage temperatures. Survival rates were found to be similar

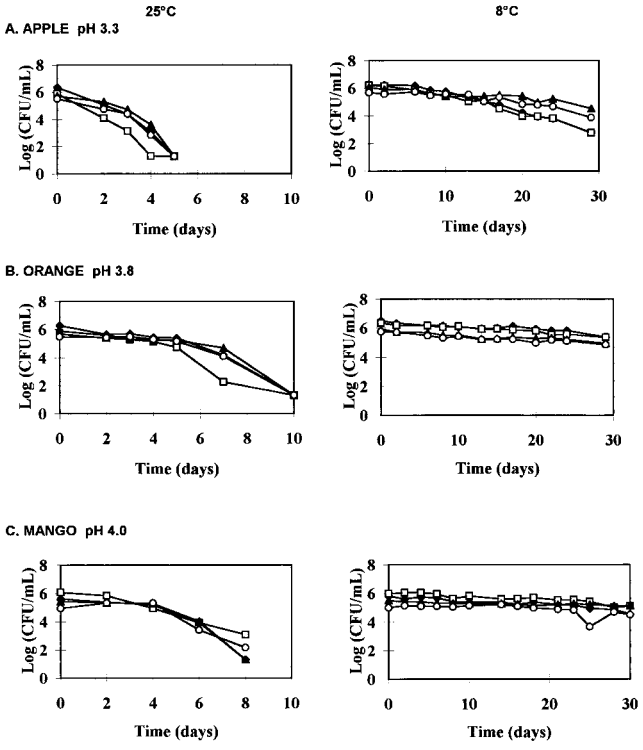

FIG. 1. Survival of *E. coli* MG1655 (◆) and the pressure-resistant mutants LMM1010 (□), LMM1020 (▲), and LMM1030 (○) in apple (A), orange (B), and mango (C) juices stored either at 8°C for up to 30 days or at 25°C for up to 10 days. The initial population in each case was between 10^5 and 10^6 CFU/ml, and the detection limit was 20 CFU/ml.

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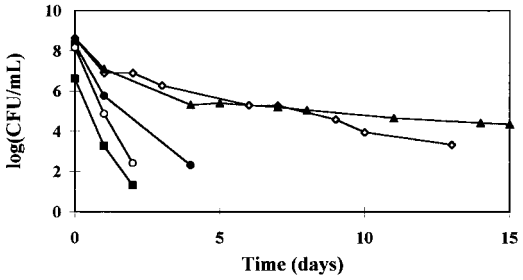

FIG. 2. Survival of *E. coli* LMM1010 in fruit juices stored at 8°C for up to 15 days after pressure treatment for 15 min at 20°C. Treatments were as follows: orange (pH 3.8), 300 MPa (◇); apple (pH 3.3), 300 MPa (■); and mango (pH 4.0), 300 MPa (▲), 400 MPa (●), or 500 MPa (○). The initial number of cells in each case was between 1.0×10^9 and 1.8×10^9 CFU/ml, and the detection limit was 20 CFU/ml.

for all strains, and they correlated positively with juice pH and negatively with temperature (Fig. 1). Based on these results, considerable survival of organisms over a period of several days or weeks can be anticipated also with lower, more realistic levels of initial contamination, depending on the juice and the storage temperature. Others have also observed that refrigeration enhances survival of *E. coli* in acidic environments (3, 4, 14); this may be due to a reduced permeability of the cell membrane to protons and/or a reduced metabolic activity.

Previously, pressure resistance of the mutants had been studied only in potassium phosphate buffer, pH 7.0 (5). To test pressure resistance at low pH, we subjected all of the strains to treatments of 300 and 400 MPa in apple and orange juices and compared the results with those obtained in HEPES buffer, pH 7.0 (Table 1). The results indicate that the mutants lose some of their pressure resistance in the juices but remain considerably more pressure resistant than the parent strain and that they have the potential to survive typical pressurization conditions that efficiently eliminate normal, non-pressure-resistant *E. coli* strains from fruit juices. For instance, 15 min at 20°C and 300 MPa caused a $>10^4$-fold reduction in the number of strain MG1655 organisms in apple juice but only a 13-fold reduction in the number of strain LMM1010 organisms. Because LMM1010 was found to be the most resistant of the strains in HEPES buffer, pH 7.0, as well as in both juices, further work was carried out only with this strain. The inactivation of LMM1010 in HEPES buffers of pH 3.0 to 7.0 (Table 1) suggests that the increased pressure sensitivity of the mutants in the juices compared to that in HEPES buffer, pH 7, is at least partly due to the low pH.

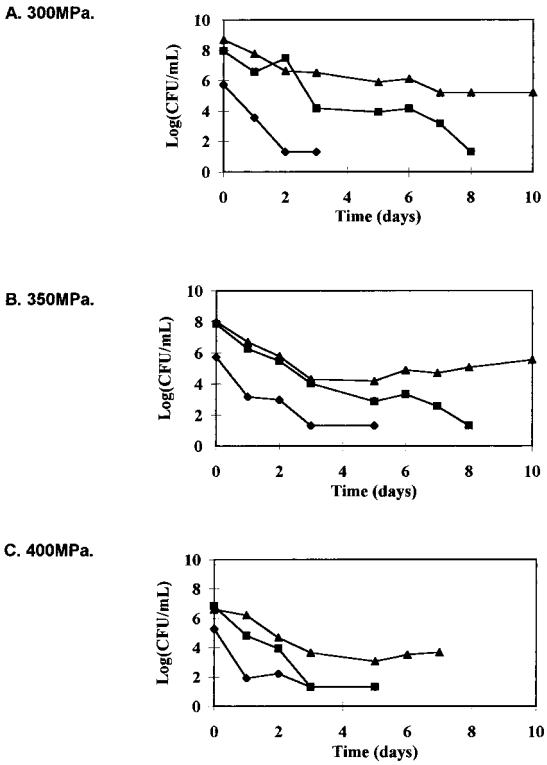

FIG. 3. Survival of *E. coli* LMM1010 in pH 3.0 (◆), pH 3.5 (■), or pH 4.0 (▲) HEPES buffer upon storage at 8°C for up to 10 days after pressure treatment for 15 min at 20°C and 300 MPa (A), 350 MPa (B), or 400 MPa (C). The initial number of cells in each case was between 0.35×10^9 and 1.8×10^9 CFU/ml, and the detection limit was 20 CFU/ml. Inactivation in non-pressure-treated controls was always <1 log after 10 days of incubation.

In the next set of experiments, we studied the long-term survival of strain LMM1010 bacteria following a relatively mild pressurization in the three juices and in low-pH HEPES buffers. The orange and apple juices were treated at 300 MPa only, while the mango juice was also treated at 400 and 500 MPa because a lower kill rate was expected in this juice due to its higher pH. At least 10^6 CFU of survivors per ml was counted

TABLE 1. High-pressure inactivation of *E. coli* parental strain MG1655 and three pressure-resistant mutant derivatives in HEPES buffer and in apple and orange juices

Pasteurization medium and pH	Inactivation of strain at pressure of[a]:							
	300 MPa				400 MPa			
	MG1655	LMM1010	LMM1020	LMM1030	MG1655	LMM1010	LMM1020	LMM1030
HEPES buffer								
pH 7.0	4.9	0.4	0.6	0.4	7.0	0.6	2.1	0.8
pH 4.0	ND[b]	0.5	ND	ND	ND	2.6	ND	ND
pH 3.5	ND	1.2	ND	ND	ND	2.3	ND	ND
pH 3.0	ND	2.9	ND	ND	ND	3.3	ND	ND
Apple juice, pH 3.3	>4.4	1.1	2.0	>3.4	>4.4	>4.7	>3.4	ND
Orange juice, pH 3.8	3.5	0.8	0.4	1.6	>4.4	1.5	2.4	>3.4

[a] Inactivation performed for 15 min at 20°C and is expressed as $\log(N_0/N)$, with N_0 and N being the counts for the untreated control and the pressure-treated sample, respectively.
[b] ND, not determined.

in each juice immediately after pressure treatment (Fig. 2). However, a considerable decrease in numbers of survivors was noticed upon storage of the pressure-treated juices at 8°C, in several cases resulting in undetectable levels after 5 days. Comparison of the results obtained with different juices at the same pressure (300 MPa), or in the same juice (mango) at different pressures, revealed that the inactivation rate during storage was inversely correlated with the juice pH and positively correlated with the applied pressure. The same conclusion could be drawn from the experiments in HEPES buffers (Fig. 3). This secondary inactivation achieved during storage was considerable compared to the primary inactivation caused by the pressurization, even under the mildest conditions. For instance, the low-level (1.1-log) direct reduction of LMM1010 organisms in apple juice by a 15-min treatment at 20°C and 300 MPa was followed by an extensive (almost 5-log) further reduction during the first 2 days of storage (Fig. 2). This phenomenon illustrates that the pressure treatment caused sublethal injury to a large proportion of cells, resulting in a reduced resistance to low pH.

From a safety standpoint, a reassuring observation is that even under the mildest conditions applied in this study (mango juice, pH 4.0, and HEPES buffer, pH 4.0; 300 MPa), a 100-fold further reduction of pressure-resistant *E. coli* organisms took place during the first 3 days of refrigerated storage (Fig. 1 and 2). Since enterohemorrhagic *E. coli* strains have a low infectious dose, this reduction may not be sufficient to provide the desired level of safety, and a higher pressure would be recommended in this particular case. Let us assume that an acceptable safety level requires a 10^5-fold reduction in numbers of organisms; this can be achieved, even in a pH 4 juice, within 2 days of refrigerated storage after a moderate pressure treatment (500 MPa, 20°C, 15 min), as is evident in Fig. 2. In other words, the observation of a 2-day quarantine period between pressure treatment and consumption may significantly increase the safety of pressure-pasteurized fruit juices.

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